Journal of Ethnopharmacology 123 (2009) 155–162

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Pharmacological investigation on the wound healing effects of Radix Rehmanniae in an animal model of diabetic foot ulcer

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ARTICLE INFO

Article history:
Received 3 June 2008
Received in revised form 4 January 2009
Accepted 2 February 2009
Available online 14 February 2009

Keywords:
Radix Rehmanniae
Rehmannia glutinosa
Diabetic foot ulcer
Chinese medicine
Wound healing

ABSTRACT

Ethnopharmacological relevance: Radix Rehmanniae (RR) has a very long history of usage in traditional Chinese medicine and is usually one of the principal herb found in many herbal formulae used in diabetic foot ulcer.
Aim of the study: RR aqueous extract was investigated for its wound healing effects in a diabetic foot ulcer rat model and its detailed mechanism of actions.
Materials and methods: A previously established diabetic foot ulcer rat model was used to assess the effect of RR extract on wound area reduction, tissue regeneration and angiogenesis. Carrageenan-induced inflammation rat model was used for inflammation study; and diabetic control was evaluated using a neonatal streptozotocin-induced diabetic rat model.
Results: In the RR treated group, a trend of reduction of the wound area was observed from days 8 to 18 and a significant difference (as compared with control group) was found on day 8. The ulcer healing effect of RR extract was further supported by better developed scars and epithelialization as well as good formation of capillaries with enhanced VEGF expression. Carrageenan-induced inflammation was also significantly alleviated with RR extract.
Conclusions: Our results demonstrated for the first time that Radix Rehmanniae was effective in promoting diabetic foot ulcer healing in rats through the processes of tissue regeneration, angiogenesis and inflammation control, but not glycemia control. The present study provided scientific basis to support the traditional use of Radix Rehmanniae in diabetic foot ulcer.

1. Introduction

Radix Rehmanniae (RR) has a long history of usage in traditional Chinese medicine (TCM). The first record appeared in the Canon on Medicinal Herbs by the Divine Ploughman (Circa 200 B.C.) *Shen-nong Bencao Jing*. It was classified as high-grade (very safe) medicine. RR is derived from the root of *Rehmannia glutinosa* Libosch. (family Scrophulariaceae) and its unprocessed form is called "Sheng Dihuang". RR has been applied in many TCM applications for wound healing. According to the classical TCM interpretation, RR can reduce heat in blood, nourish yin and promote the production of body fluid (Yen, 1997). Based on such theory, the herb would facilitate the healing of chronic ulcers through the elimination of the "yin" deficiency, detoxification, promotion of blood flow and removal of pathogenic heat which are symptoms commonly found in diabetic patients with foot ulcers. As a result, RR is used in many traditional herbal formulae for foot ulcer such as "Cleansing Stagnant Blood from the Mansion of Blood Decoction", "Four Powerful Herbs Decoction", and "Heat-clearing and Yin-enriching Decoction".

Classic TCM references have described the application of RR in the healing of foot ulcers (Commission of the State Administration of Traditional Chinese Medicine of the People's Republic of China, 1999). For example, RR has been used in the application of enriching bone marrow and producing muscle tissue according to the Canon on Medicinal Herbs by the Divine Ploughman (Circa 200 B.C.) *Shen-nong Bencao Jing*. This traditional practice may be interpreted as granulation formation and tissue regeneration in modern interpretation. Besides, according to the Alternate Records of Famous Physicians (Tao Hongjing 452–536) *Mingyi Bielu*, the herb could promote the circulation of "qi" and blood that could be explained currently as "angiogenic". The herb was also applied in reducing pain from inflammation in the lower limb, according to the information of the Pandects of Natural History (Li Shizhen 1518–1593)

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doi:10.1016/j.jep.2009.02.010

Bencao Gangmu. Our recent study also demonstrated similar foot ulcer healing effects of RR aqueous extract in diabetic rats (Lau et al., 2008). The correlations between traditional TCM experience and modern scientific studies have inspired us to search for the mechanistic explanation for the effects of RR in diabetic foot ulcer healing.

Diabetic neuropathy and/or vasculopathy are the main determinants of diabetic foot ulcer, which involves very complicated mechanisms of wound healing. The healing process is conventionally divided into several stages: (1) inflammation, (2) granulation formation, and (3) tissue remodeling (Singer and Hollander, 2003). Some other pathology hindering wound healing under diabetic conditions include impaired angiogenesis and high blood glucose level (Mason et al., 1999; Chabbert-Buffet et al., 2003; Martin et al., 2003).

With the previous promising results obtained with RR extract in terms of promotion of wound healing in the diabetic foot ulcer animal model (Lau et al., 2008), the main aim of the present study was to further investigate the wound healing effect of RR extract using the same animal model, and the detailed mechanistic studies including tissue regeneration, angiogenesis, plasma glucose level and inflammation control.

2. Materials and methods

2.1. RR authentication and extract preparation

The raw herb RR was purchased from a herbal shop in the wholesaler market in Hong Kong. The herb was morphologically authenticated by a botanical expert, and its thin layer chromatographic profile was also compared according to the Pharmacopoeia of the People's Republic of China, 2005 edition (Chinese Pharmacopoeia Commission, 2005). Voucher specimen of RR was deposited in the museum of the Institute of Chinese Medicine, the Chinese University of Hong Kong with voucher specimen number 2003-2452.

For extract preparation, 500 g of the sliced herb was boiled twice in 2.5 L of distilled water for 2 h under reflux. The supernatant was filtered through cheese cloth and then subjected to freeze-drying (Thermo Savant MODULYO freeze-dryer, E-C Apparatus Corp., Holbrook, NY, USA). The dosage of the freeze-dried powder used for both animal studies was 1.85 g/kg. It was calculated basing on the maximum human equivalent dosage of 90 g raw herb. The freeze-dried powder was reconstituted in distilled water prior to treatment. The RR extract was force-fed by gavage to the rats.

2.2. Animals

Female albino Wistar rats were used in ulcer and diabetic studies and female Sprague Dawley rats were used in inflammation studies. All rats were supplied by and kept in the Laboratory Animal Service Centre, The Chinese University of Hong Kong. Four rats were kept in a wire-bottomed cage, in a room with 12-h light–dark cycle and 22–25 °C. Normal rodent diets (Prolab 2500 rodent diet) and tap water were constantly supplied to the rats. Bedding under the cage was changed at least twice a week.

The animal experiments were conducted with the licence under Animals (control of experiments) Ordinance (Cap. 340) issued by Department of Health of the Hong Kong Government; and according to the guidelines of Animal Experimentation Ethics Committee, the Chinese University of Hong Kong.

2.3. Diabetic foot ulcer study

2.3.1. Ulcer area measurement

The induction of diabetes and foot ulcer on Wistar rats was consistent with the procedure in our previous publication (Lau et al., 2008). In brief, the adult diabetic rats (>300 mg/dL) were used for wound induction. On the day of wound induction (defined as day 0), each rat was anesthetized with an intraperitoneal injection of 75 mg/kg ketamine and 10 mg/kg xylazine. A rectangular pattern was marked on the dorsal surface of the foot using a signet, then a layer of skin in full thickness with standard area of 2 mm × 5 mm was removed. The initial wound size would be measured on day 1.

Planimetric measurements were performed on digital photographs taken from each rat's foot and the pictures were analyzed using an Image Analysis Computer Program (IACP). Twenty-four bit true color images of the rat's foot were captured using a digital camera [Nikon D100, Nikon, Japan] positioned 20 cm above a graphics table. Each digital image was taken with a horizontal and vertical resolution of 1504 and 1000 pixels, respectively. Prior to picture taking, the leg of the rat was gently pinched manually so that the ulcer site was positioned at the intersection of the vertical and horizontal grid lines on the graphics table. All digital images were captured by the same investigator.

The captured digital images were deconstructed into their primary components using specifically written PASCAL language Image Analysis Computer Program (Borland Delphi 6.0, Borland Inc., USA). IACP employed conventional image analysis techniques such as color thresholding, edge detection and color filtering. The ulcer photo was then further segmented into three regions, either "raw wound", "new epithelialization" and "undamaged intact skin" regions using the color gradient differences between adjacent pixels. The area of the ulcer (mm^2) was determined by counting the number of pixels contained within the region bound by the wound edge.

A total of 54 rats were used in this animal study and they were equally divided into control (water) and RR extract treatment groups, i.e. 27 rats in each group. Ulcer photographs were taken on days 1, 4, 8, 13 and 18. Multilevel statistical model was employed to compare the rate of ulcer area reduction on days 4, 8, 13 and 18 relative to the baseline on day 1. Such model could be used to control the variation among the rats and detect the significant differences in ulcer area reduction rates between the RR extract group and the water group. The public domain software *MIXREG* (as accessed by http://tigger.uic.edu/~hedeker/mix.html) was employed for fitting multilevel model and all the other statistical analyses were performed using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). All statistical tests were two-sided, with a significant level of $p < 0.05$.

2.3.2. Tissue regeneration study

Observations on wound surface provided information on the gross extent of wound healing. Since a lot of physiological activities and physical changes underneath the skin were equally significant in ulcer healing, epithelialization and tissue regeneration such as scar formation and granulation formation were carefully observed in this animal study.

The granulation tissue of 18 rats randomly selected from either the RR extract group or water group in the diabetic foot ulcer animal studies were collected and preserved in 10% formalin (1000 mL of 0.1 M phosphate buffer + 100 mL of formaldehyde (37%)) for fixation. Standard methods were then used for sectioning and staining with hematoxylin and eosin (H&E) (Berezovski, 1978). Photographic images were taken by Zeiss Spot Camera (Carl Zeiss, Inc.). The development of scar, epidermis and granulation tissues was inspected.

2.3.3. Angiogenesis study

In the studies of angiogenesis, the number of capillaries was counted in the granulation tissues sectioned with hematoxylin and eosin staining. Immunohistochemical methods were also used to determine the release of vascular endothelial growth factor (VEGF) in the ulcer area. VEGF is a proangiogenic cytokine widely used to assess the formation of new blood vessels during wound repairs

(Leung et al., 1989; Brown et al., 1992; Altavilla et al., 2001; Elcin et al., 2001; Romano Di Peppe et al., 2002; Galeano et al., 2003; Galiano et al., 2004).

Hence, the ulcer granulation tissue with H&E stains was further examined at high magnification (200×) in order to count the number of capillary sprouts in the granulation. All samples from the same 18 rats were counted by the same investigator in a blinded manner. The number of capillary sprouts was compared between the treatment groups of water and RR extract by Mann–Whitney test. Furthermore the granulation tissues of these rats were stained for VEGF(147) antibody (ImmunoCruz Staining Assay, Santa Cruz, CA, USA) with an aim to detect the *in vivo* VEGF release from the implanted microspheres. They were examined on days 4, 8, 13 and 18. The immunostaining system is based on an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to an amino acid sequence mapping at the amino-terminus of VEGF (165 amino acid spice variants) of human origin. The intensity of the VEGF expression was compared between RR extract and water treatment groups.

2.4. Plasma glucose study

Plasma glucose control is an important factor in the healing process of diabetic foot ulcer. Since type 2 diabetes mellitus contributes to 95% of foot ulcer cases, relevant experimental models should be used for the study. In our present study, a neonatal streptozotocin (STZ)-induced diabetic Wistar rat model was used to achieve hyperglycaemic condition (Kergoat et al., 1991; Melin et al., 1991). A total of 50 adult n5 (5 days after birth) STZ-induced diabetic rats were used as an *in vivo* diabetic model. Basal glycemia test was performed to characterize the effects of RR extract on the regulation of plasma glucose. Glucose determination kit and 100 mg/dL (5.56 mM) glucose standard were purchased from BioSystems (Barcelona, Spain).

For the basal glycaemia test, the experimental procedures were the same as described in our previous studies (Chan et al., 2007). Fifty diabetic rats were randomly divided into three groups: negative control (water, 5 mL/kg body weight), positive control (metformin, 200 mg/kg), and treatment group (RR extract at dosage of 1.85 g/kg body weight). The experiment lasted for eight consecutive days (days 1–8). Treatments were given to the rats every day and plasma glucose levels were determined on day 1 (for studying the short-term effect of RR extract), and on day 8 (for studying the long-term effect). Multilevel statistical model was employed to compare the differences of glucose level at each time point (t = 45, 90, 135, 180, 240, 360 min) relative to baseline (t = 0) among the different treatment groups (water, metformin and RR extract) on each day (days 0, 1 and 8). Moreover, the sum of differences ($\Sigma\Delta G$) of plasma glucose was calculated by summation of the differences between the plasma glucose level at each time point and that of time point 0. The $\Sigma\Delta G$ values for the treatment groups were then compared with those from the water group using Student's t-test with a significant level of $p < 0.05$.

2.5. Inflammation study

Inflammation is one of the critical steps in wound healing and may affect the other phases of ulcer healing especially during granulation formation (Shekhter et al., 1984; Collier, 2003). However, improper inflammation regulation might cause harm to wound healing (Park and Barbul, 2004; Martin and Leibovich, 2005). In this study, carrageenan-induced paw edema in rats, a widely adopted model for investigating inflammation regulatory activities of raw herbs or drugs, was used (Winter et al., 1962; Stefanova et al., 1995; Olajide et al., 1999; Lam and Ng, 2003).

A total of 60 female Sprague Dawley rats of 250–300 g were used in this study. They were first anesthetized with thiopentone (40 mg/kg). All inflammation induction was carried out using carrageenan injection in standard procedures (Winter et al., 1962; Lam and Ng, 2003). Thirty rats were randomly placed into each group which was treated with water (as control) or RR extract (1.85 g/kg). Each rat received treatment three times at 8 h interval after inflammation induction. With an aim of assessing the degree of swelling and inflammation, three parameters: knee joint size, articular blood flow and plasma extravasation, were measured 24 h after inflammation induction.

2.5.1. Knee joint size

The knee joint sizes of both legs of each rat were measured by a micrometer before carrageenan injection. The knee joint size at this time point was considered as baseline value which was compared with those at 24 h after injection. Multilevel statistical model was used for data analysis. All statistical tests were two-sided, with a significant level of $p < 0.05$.

2.5.2. Articular blood flow

Twenty-four hours after the injection of carrageenan or saline, the rats were deeply anesthetized with urethane (1.8 g/kg). A laser Doppler perfusion imager (Moor Instruments, UK) was used to measure knee joint blood flow at red (633 nm) and infra-red (780 nm) wavelengths. The detected images were then analyzed by a software namely, Laser Doppler Perfusion Imaging Programme. ANCOVA statistical model was used for data analysis with a significant level of $p < 0.05$.

2.5.3. Plasma extravasation

Twenty-four hours after carrageenan or saline injection, Evans blue (50 mg/kg) was injected into the jugular vein of the rats. Anterior and posterior portions of the knee joint capsule were dissected and collected from both legs. Evans blue content in the synovial tissue was extracted and measured spectrophotometrically at a wavelength of 620 nm (Biochrom 4060). ANCOVA statistical model was used for data analysis with a significant level of $p < 0.05$.

3. Results

3.1. RR authentication and extraction

RR was authenticated using its morphological characteristics and also chemical analysis using thin layer chromatography. Catalpol was used as a chemical reference marker for authentication according to the Pharmacopoeia of the People's Republic of China, 2005 edition (data not shown). The species of herb used in our study was confirmed to be *Rehmannia glutinosa*. The aqueous extract of RR was prepared and the extraction yield was 50% (w/w).

3.2. Diabetic foot ulcer study

3.2.1. Ulcer area measurement

The wound healing effect of RR extract was compared with the water treated group on days 4, 8, 13 and 18 in the diabetic rat foot ulcer model. On day 1, the baseline wound areas of water group and RR extract group were similar, 26.7 mm^2 and 26.9 mm^2, respectively with no statistical difference ($p = 0.847$, Fig. 1). A trend of greater reduction of the ulcer area from days 8 to 18 was observed in the RR extract group, with a statistical significant difference on day 8 as compared to the water treated group. Moreover, nearly all the rats in the RR extract group had ulcers completely healed on day 18. Our results were similar to those of our previous study (Lau et al., 2008), though the previous study focused more on the effect of RR extract on day 8; whereas our present study looked at a longer wound healing profile.

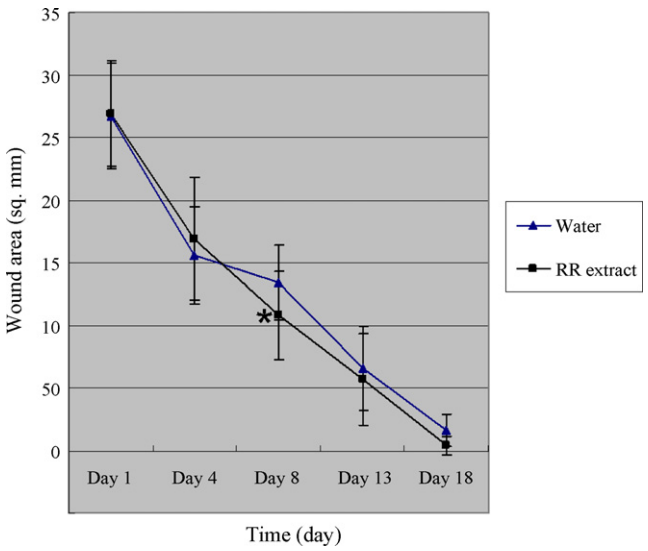

Fig. 1. Effect of Radix Rehmanniae (RR) extract on wound area (mm^2) with standard deviation from days 1 to 18 ($n = 54$). Diabetic foot ulcer animal model was adopted for testing the wound healing effects of RR extract (1.85 g/kg) over 18 days. Multilevel statistical model was employed to compare the rate of wound area reduction on days 4, 8, 13 and 18 relative to the baseline on day 1. There was a trend of bigger reduction of wound area from days 8 to 18 in the RR extract group. As indicated by the p-value of 0.044, a significant difference was found on day 8. $^*p < 0.05$ using Wilcoxon signed rank tests.

3.2.2. Tissue regeneration

Using the specimens with H&E staining, randomly selected photographs of wound tissue from rats treated with water or RR extract are shown in Fig. 2. In general, the epithelialization and scar formation were not well developed under water treatment, whereas they were well developed in RR extract treated group. Furthermore, the granulation tissue in the RR extract group was also more solid. It implied that RR extract might have improved wound healing by facilitating tissue regeneration.

3.2.3. Angiogenesis study

The number of capillaries in the granulation area was counted and the general phenomenon in each group was illustrated in Fig. 3. Using Mann–Whitney test, the median number of blood vessels in the water group was 10 which is significantly smaller than 28 in the RR extract group ($p = 0.003$).

Based on the study of VEGF secretions, it was found that the rats treated with RR extract had prominent VEGF expression starting from day 4 and such expression lasted until day 13 as shown in Fig. 4a, c and e. However those rats treated with water only began to have moderate VEGF expression from day 8 till day 18 as shown in Fig. 4d, f and h. Our results demonstrated that RR extract could enhance the VEGF expression in an earlier stage of healing on day 4, which might result in enhanced angiogenesis and better wound healing as observed in the RR extract treated group.

3.3. Plasma glucose study

Metformin was used as a positive control in our study. The significant sugar lowering effect of metformin validated our diabetic animal model. The short-term effect of RR extract on plasma glucose control, as demonstrated by the responses of basal glycemic test on day 1 (Fig. 5a), only showed statistical significance at time point of 360 min. However the treatment effects at the same time point was not significant after 7 days of RR extract treatment, while only a trend of decrease in the plasma glucose was observed (Fig. 5b). The sum of differences ($\Sigma \Delta G$) of plasma glucose in RR extract group were 481.9 mg/dL on day 1 and 759.1 mg/dL on day 8 which were not

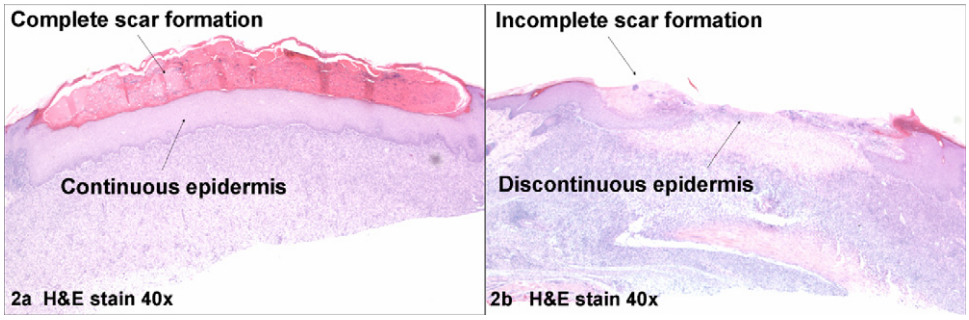

Fig. 2. Effects of Radix Rehmanniae (RR) extract on tissue regeneration on day 8 ($n = 18$). Standard methods were used for sectioning and staining with hematoxylin and eosin (H&E). Two representative figures were used to illustrate the wound healing progress in two different treatment groups: (a) wound tissue from RR extract group showed complete scar formation and continuous epidermis; (b) wound tissue from water group showed incomplete scar formation and discontinuous epidermis.

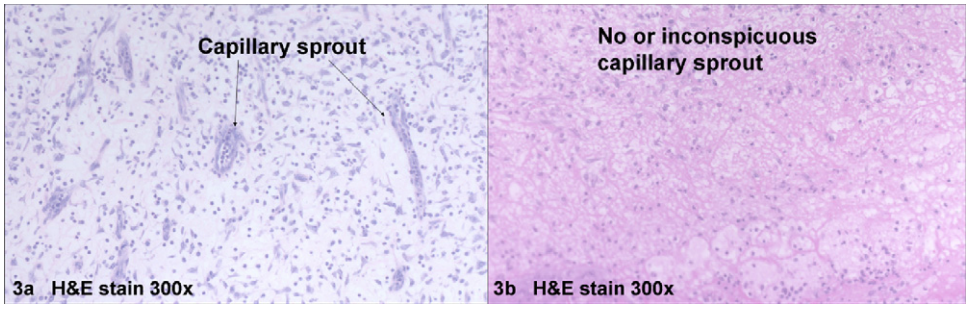

Fig. 3. Effects of Radix Rehmanniae (RR) extract on angiogenesis on day 8 ($n = 18$). The wound tissue with H&E stains was examined in granulation area. Two representative figures were used to show the phenomenon of angiogenesis in the RR extract or water treatment groups on day 8: (a) RR extract showed prominent capillary sprout in granulation tissue; (b) Water treatment group has no or inconspicuous capillary sprout.

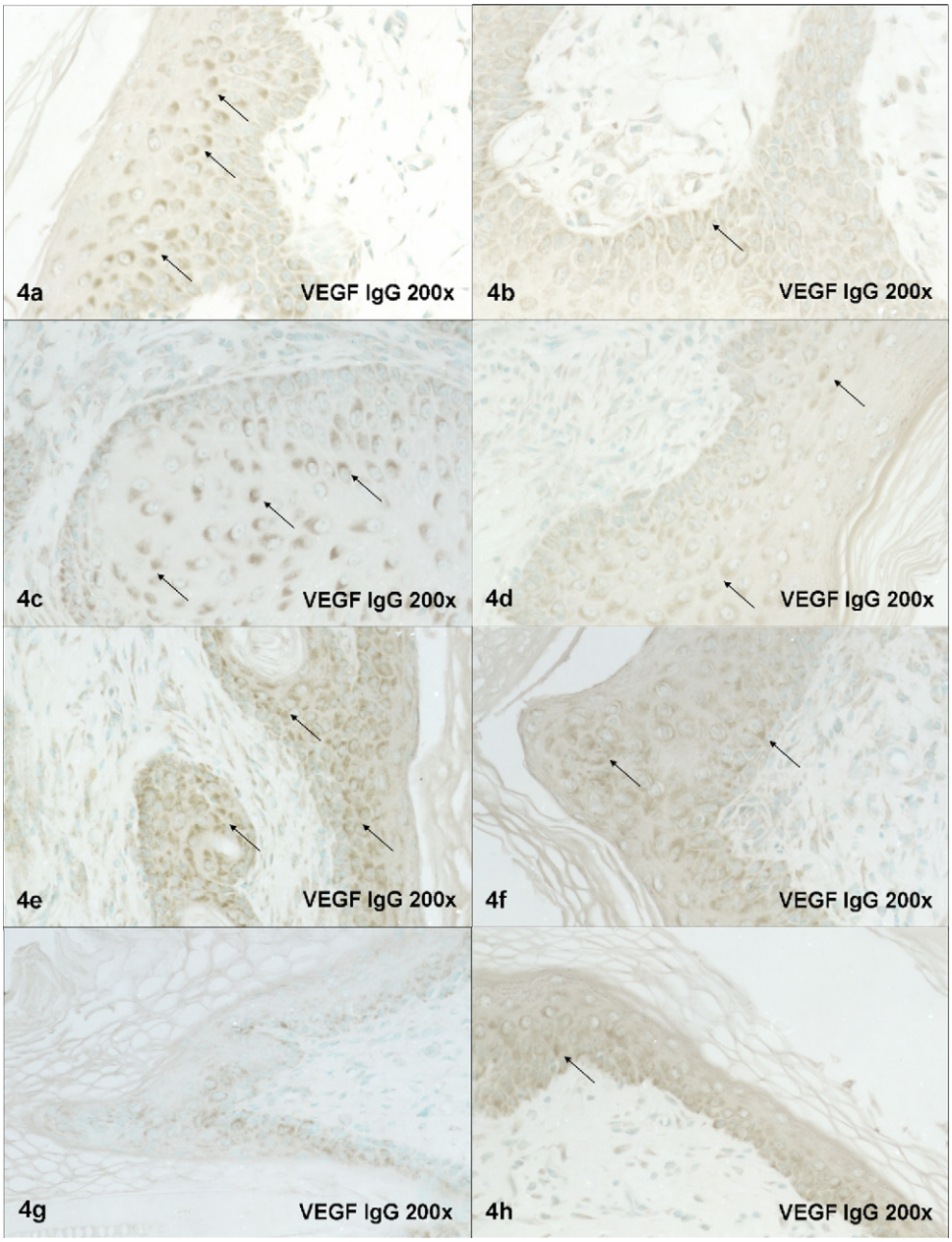

Fig. 4. Effects of Radix Rehmanniae (RR) extract on VEGF expression from days 4 to 18. VEGF (147) antibody was used to detect VEGF expression from epidermis. The intensity of brown stains in the cytoplasm of epidermal cells represented the level of the VEGF expression. A series of staining was performed from days 4 to 18 in the treatment group of water or RR extract. (a) Strong expression of VEGF IgG in wound epidermis of diabetic rat with RR extract on day 4. (b) Weak expression of VEGF IgG in wound epidermis of diabetic rat with water treatment on day 4. (c) Strong expression of VEGF IgG in wound epidermis of diabetic rat with RR extract on day 8. (d) Moderate expression of VEGF IgG in wound epidermis of diabetic rat with water treatment on day 8. (e) Strong expression of VEGF IgG in wound epidermis of diabetic rat with RR extract on day 13. (f) Moderate expression of VEGF IgG in wound epidermis of diabetic rat with water treatment on day 13. (g) Weak expression of VEGF IgG in wound epidermis of diabetic rat with RR extract on day 18. (h) Moderate expression of VEGF IgG in wound epidermis of diabetic rat with water treatment on day 18.

statistically different from those in water group with 348.8 mg/dL on day 1 and 571.2 mg/dL on day 8 (p-values = 0.316 on day 1; 0.413 on day 8). However, a greater difference ($\Sigma\Delta G$) of plasma glucose between RR extract group and water group was found on day 8, though it was not statistical significant. Our results suggested that RR is not a potent agent for plasma glucose control and that the wound healing effects of RR might be independent of glycaemia control.

3.4. Inflammation study

Under the effects of carrageenan injection, the knee size increased from 9.9 to 10.5 mm in the RR extract group; however the knee size in the control group increased to a larger extent from 9.8 mm to 11.9 mm as shown in Table 1. The size increment of the knee in the RR extract group was significantly lower than that in the water group ($p < 0.001$). These results showed that RR extract had significant effects in reducing the swelling of rat's knee with carrageenan-induced inflammation. As for the articular blood flow, the RR extract group showed significantly smaller average blood flow than that in water group by red or infra-red detection (Table 1). In addition, the mean Evans blue extravasation of the knee was 134.4 μg/g in the RR extract group which was significantly smaller than 195.1 μg/g in the water group. Hence RR extract significantly reduced plasma extravasation of the knees with carrageenan-induced inflammation.

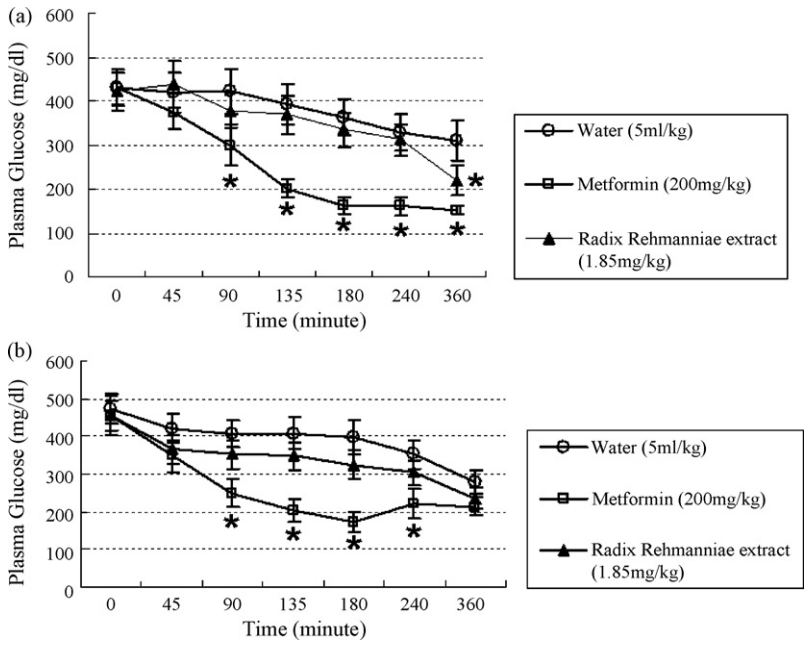

Fig. 5. Effects of Radix Rehmanniae (RR) extract on diabetic control on days 1 and 8. Basal glycaemia test was performed to characterize the effects of RR extract on the regulation of plasma glucose. Glucose determination kit and 100 mg/dL (5.56 mM) glucose standard were used. (a) Treatment response at different time points from 0 to 360 min on day 1 (short-term effects). (b) Treatment response at different time points from 0 to 360 min on day 8 (long-term effects). Data are expressed as mean ± S.D. (*$p < 0.05$; $n = 50$; by multilevel statistical model).

Table 1
Effects of Radix Rehmanniae (RR) extract on inflammation control. Carrageenan-induced inflammation rat model was used to assess the anti-inflammatory effects of RR extract. Results showed that RR extract significantly reduced the syndrome of inflammation ($n = 60$; by multilevel or ANCOVA statistical model). Mean ± standard deviation of knee joint size, articular blood flow and plasma extravasation of the water and RR extract treatments are shown in the table.

		Water	Radix Rehmanniae (RR) extract	*p*-value
Knee joint size (mm)	Before carrageenan injection	9.8 ± 0.3	9.9 ± 0.3	<0.001**
	After carrageenan injection	11.9 ± 1.1	10.5 ± 0.5	
Articular blood flow (Perfusion units: flux)	Red detection	616.9 ± 417.2	299.0 ± 150.4	<0.001**
	Infra-red detection	238.7 ± 169.1	77.4 ± 34.4	<0.001**
Plasma extravasation (amount of Evans blue dye; μg per gram of synovial tissue)		195.1 ± 113.6	134.4 ± 43.8	0.029*

* $p < 0.05$.
** $p < 0.01$.

4. Discussion

The time of ulcer area determination was based on the time profile previously adopted for wound healing studies with a significant change reported (usually on day 7 or 8) (Fahey et al., 1991; Rendell et al., 1996; Mizuno et al., 2002). In our present study, a significant difference was found on day 8 as indicated by the *p*-value of 0.044. Our histological studies also showed that a general trend of better epithelialization and scar formation as well as granulation maturation was found in the RR extract treated group after 7 days of treatment.

Diabetes has been shown to inhibit angiogenesis (Bek et al., 2002; Da Costa Pinto and Malucelli, 2002). On the other hand, a generalized microangiopathy could reduce nutrient supply to the ulcer tissues and lead to impaired diabetic foot ulcer healing (Chabbert-Buffet et al., 2003). Therefore angiogenesis is important in diabetic foot ulcer healing. By looking at the capillary number in the granulation tissues, more capillaries were found in the RR extract group on day 8; this was further supported by the observation of enhancement in the expression of VEGF from epidermal cells earlier on from day 4. VEGF is a potent growth factor for angiogenesis and essential in wound healing (Leung et al., 1989; Brown et al., 1992; Altavilla et al., 2001; Elcin et al., 2001; Romano Di Peppe et al., 2002; Galeano et al., 2003; Galiano et al., 2004). Our findings demonstrated that epidermis was mainly responsible for the VEGF expression which is consistent with the findings of other research groups (Brown et al., 1992).

Radix Rehmanniae was described to have hypoglycemic effects from traditional practice of Chinese medicine, and was also observed from some other scientific research studies (Kiho et al., 1992; Miura et al., 1997). However, in this study, RR extract was only associated with a drop of plasma glucose at one time point after single treatment, probably due to the fact that time is required for the digestion and absorption of RR. A greater trend of decrease in plasma glucose in the long-term treatment of RR was observed. This suggested a possible cumulative effect of RR. Besides, based on our previous studies using HPLC-ELSD (Lau et al., 2007), RR extract was found to contain 5.8% (w/w) fructose, 4.8% (w/w) glucose and 7.1% (w/w) sucrose. In the present study, a rat of an average weight of 270 g under a dosage of 1.85 g/kg RR extract would have therefore consumed 0.029 g fructose, 0.024 g glucose and 0.035 g sucrose, which gives a total of 0.088 g of sugar. Assuming that all these sugars were absorbed and digested to glucose, and an estimated rat plasma volume of 15 mL, the daily administration of RR extract would instantly increase the plasma glucose by 587 mg/dL. Hence the high sugar content of RR extract might possibly affect

the basal plasma glucose and the glucose-lowering effect of the herb.

For the quantification of the state of inflammation, we have adopted the carrageenan-induced inflammation animal model (Lam and Ng, 2003). In the measurement of articular blood flow, all rats had to be deeply anesthetized with urethane. Under unconsciousness, rats are likely to be in an unusual physiological state (Bibby and Grimble, 1988; Westmoreland et al., 1991; Iltis et al., 2005), and the state of hyperemia induced by carrageenan might not be uniform in all animals. This might explain the large variations in the values of blood flow as shown by the large standard deviation. Nonetheless, RR extract still elicited a strong effect in suppressing carrageenan-induced hyperemia to near the level of normal knee joint blood flow. The mechanisms of blood flow response in wound healing may be more complex than the simple inflammatory vasodilatation as conventionally postulated (Rendell et al., 1996; Rendell et al., 2002). Even so, the RR extract is not expected to reduce nutrient supply to the wound tissue beyond the normal level with its anti-hyperemic action. Moreover RR extract was found to control inflammation which could be beneficial to wound healing in general.

After the measurement of articular blood flow, the rats were immediately sacrificed for the extraction of synovial tissue from the knee joint. At the time of sacrifice, the Evans blue dye had been circulating in the blood vessels of the unconscious rats for 4 h, it was expected that more dye would diffuse into the synovial tissue of the knee as its vascular permeability increased with inflammation (Ito et al., 1995). According to Table 1, the standard deviation was quite large, implying that the change of vascular permeability in response to carrageenan or RR extract varied qualitatively (Watanabe, 1985). Last but not least, all the diffusion action of Evans blue happened under an unconscious state of the rats. We observed that the unconscious rats might not have reacted as normally as in their conscious state. All these limitations and assumptions might contribute to the variation of plasma extravasation. As inflammation involves very complicated physiological responses, more studies should be conducted to further investigate the effects of RR extract on wound healing, i.e. cytokine secretion such as TNF and interleukin may help further assess the role of RR in modifying inflammatory responses (Bechtel et al., 1996; Theze, 1999; Grellner, 2002; Tonks et al., 2003).

5. Conclusion

Our results demonstrated for the first time that Radix Rehmanniae was effective in promoting diabetic foot ulcer healing in rats through the processes of tissue regeneration, angiogenesis and inflammation control. These effects, however, seemed to be independent of blood glucose control. The present study provided scientific basis to support the traditional use of Radix Rehmanniae (usually as principal herb in herbal formulae) in diabetic foot ulcer. Future work will focus on the identification and purification of the active component(s) of Radix Rehmanniae.

Acknowledgements

The authors would like to thank Dr. K.C. Choi of the Center for Epidemiology and Biostatistics, the Chinese University of Hong Kong for his technical help in statistical analysis; Dr. H. Cao of the National Engineering Research Center for Modernization of Traditional Chinese Medicine (Zhuhai, Guangdong, China) for his help in the morphological authentication of Radix Rehmanniae; Ms. K.L. Choi of the Institute of Chinese Medicine, the Chinese University of Hong Kong for the chemical authentication of Radix Rehmanniae using thin layer chromatography; and Ms E.S.K. Ng of the Department of Pharmacology, the Chinese University of Hong Kong for her technical support in experiments on the carrageenan-induced inflammation animal model.

The work described in this paper was substantially supported by a grant from the University Grants Committee of the Hong Kong Special Administrative Region, China under the Area of Excellence project "Chinese Medicine Research and Further Development" (Project No. AoE/B-10/01) coordinated by the Institute of Chinese Medicine of the Chinese University of Hong Kong.

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